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13. ABSTRACT (Maximum 200 Words)

Erythropoietin is effective in correcting the anemia associated with cancer and chemotherapy. However, Epo receptors (EpoR) have been found on tumor cells and Epo may stimulate these cells. We discovered that prostate cancer cell lines and primary prostate tumors express EpoR. In this study, we propose to gain insight into a pattern of EpoR expression in primary human prostate tumors and adjacent normal tissue and to study the role of the EpoR and the effect of Epo administration on growth of prostate cancer cells transplanted into SCID mice. We have prepared prostate cancer cell lines containing antisense EpoR constructs to be used in loss-of-function studies in vivo. Because we had found that the tetracycline inducible vector system was very "leaky", we transfected LNCaP and PC-3 cells with stable antisense constructs. We found that the single stranded cDNA probe was not sensitive enough and, therefore, tested an S1 nuclease protection technique. We complete 50 % of our immunohistochemistry pilot study showing EpoR in primary human prostate tumors. We also determined of baseline growth kinetics for prostate cancer cells transplanted into SCID mice.

14. SUBJECT TERMS
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INTRODUCTION

Recombinant human erythropoietin is an extremely effective therapeutic agent that can correct the anemia associated with chronic renal failure. In addition, it is gaining increased use in the treatment of anemia in cancer patients with or without chemotherapy. However, there have been several reports demonstrating that Epo receptors (EpoR) are found on tumor cells and that Epo may directly stimulate their survival, growth and proliferation. We have discovered that human prostate cancer cell lines LNCaP and PC-3 as well as immortalized prostatic epithelial lines express functional EpoR. Moreover, the EpoR gene is also expressed by primary human prostate tumors. We hypothesize that endogenous Epo may serve as a previously unrecognized regulator of prostate cell biology. Moreover, the use of pharmacological doses of recombinant Epo in clinical practice, including the treatment of the anemia associated with prostate cancer, may further influence disease progression. In order to address this possibility with human prostate cancer, we have proposed to carry out a pilot study to gain insight into the pattern of EpoR expression in primary human prostate tumors and adjacent normal tissue. Additionally, by studying prostatic tumor lines expressing the Epo receptor or with the Epo receptor expression downregulated, we propose to study the role of the EpoR and the effect of recombinant Epo administration on the growth and metastasis of these prostate cancer cells transplanted into SCID mice. The results of our studies should lead to important insights into the role of endogenous and exogenous Epo in prostate cancer biology and into the use of recombinant Epo therapy in prostate cancer patients.

BODY

Task 1: Establish cell lines for loss-of-function experiments.

In the prior year, we had subcloned EpoR cDNA into the inducible expression vector Tet-On. However, we discovered that the Tet-On system was quite leaky, that is, it exhibited substantial expression of the inserted transgene even in the absence of tetracycline. We carried out a series of experiments on the Tet-On vector itself and discovered that the kanamycin resistance cassette contained a promoter sequence that activates the downstream gene, resulting in constitutive expression. We determined that deletion of this kanamycin cassette results in a highly regulatable silent vector in the absence of tetracycline induction. During this past year, we wrote a manuscript describing our findings and submitted it for publication. Next we transfected both LNCaP and PC-3 cell lines with the stable antisense constructs. Because both conventional northern blot using a double stranded cDNA probe and real time PCR failed to discriminate between expression of the sense EpoR messenger mRNA from the antisense mRNA, we have designed a single stranded cDNA probe that is specific or the endogenous EpoR mRNA. Unfortunately, this technique was insufficiently sensitive. Next, we moved forward with a S1 nuclease protection assay. It, too, proved insufficiently sensitive. We have now just completed the design of an RT-PCR method using a two-step approach with strand specific nested primers. In preliminary testing, it appears to offer the sensitivity that we require.

Task 2: To carry out a pilot study to determine the distribution of EpoR in primary tumors, metastatic foci and normal prostate tissue.

In the prior year, we used our polyclonal affinity purified rabbit anti-human EpoR antibody to optimize immunohistochemistry methods on the rodent BaF3 cell line stably transfected with the human Epo receptor and compare these results with non- transfected cells. As shown in Figure 1, cells lacking the EpoR have a relatively low background of antibody binding (brownish red pigment). In contrast, as shown in Figure 2, cells expressing the human EpoR are highly positive for antibody binding.

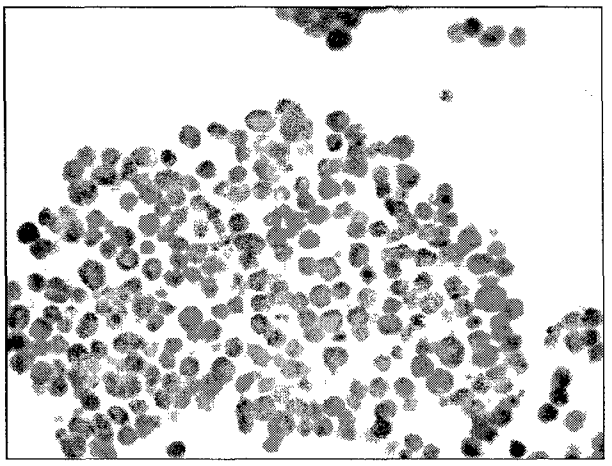

Figure 1. Control BaF3 cells stained with anti-EpoR antibody.

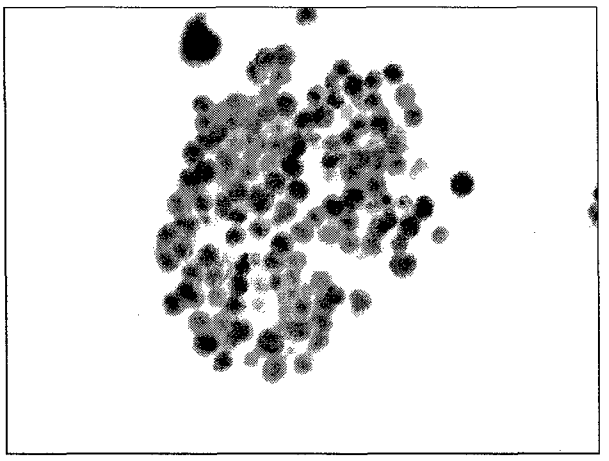

Figure 2. BaF3-EpoR cells stained positively with anti-EpoR antibody.

These conditions were then used to test for Epo receptor in human prostate cancer cell line PC-3. As shown in Figure 3, immunostaining in PC-3 was robust and heterogeneous.

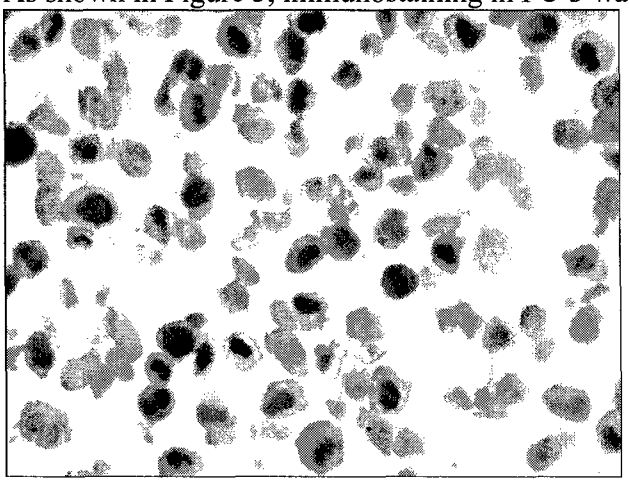

Figure 3. Robust EpoR immunostaining of PC-3 prostate cancer cells.

Finally we carried out preliminary studies of fixed and embedded human prostate cancer primary tumors. We confirmed expression of Epo receptor by malignant prostatic epithelial cells (Figure 4). These results prove that it will be possible for us to carry out all of Task 2.

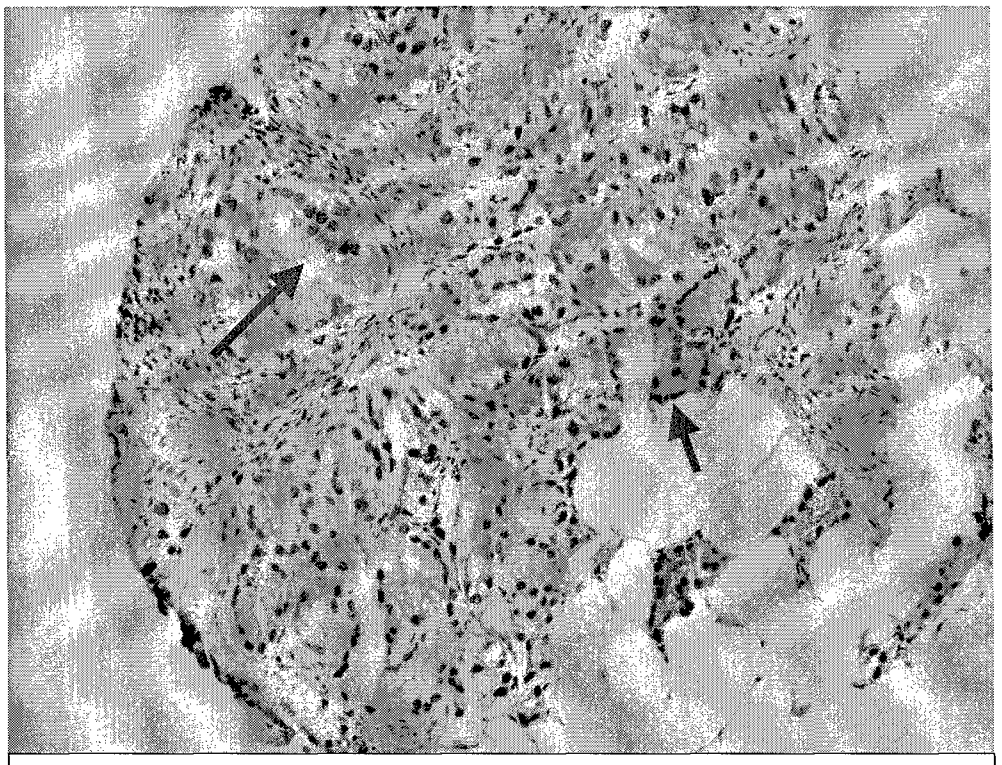

Figure 4. Positive EpoR immunostaining of malignant prostatic epithelium in a primary human prostate cancer. Red arrows point to two of many positive areas. Stroma is negative.

During the past year, we completed approximately 50 % of our proposed pilot study of EpoR expression by primary human prostate tumors.

Task 3: To study the role of the EpoR and the effect of recombinant Epo administration on the growth and metastasis of human prostate cancer cells transplanted into SCID mice.

We have completed the determination of baseline growth kinetics for nontransfected wildtype LNCaP and PC-3 cells transplanted subcutaneously into SCID mice.

KEY RESEARCH ACCOMPLISHMENTS:

- Tested single stranded DNA probe for EpoR in northern blots

- Tested S1 nuclease protection assay
- Completed the design of an RT-PCR method using a two-step approach with strand specific nested primers. In preliminary testing, it appears to offer the sensitivity that we require.
- Completed approximately 50 % of our proposed pilot study of EpoR expression by primary human prostate tumors.
- Completed the determination of baseline growth kinetics for nontransfected wildtype LNCaP and PC-3 cells transplanted subcutaneously into SCID mice.

REPORTABLE OUTCOMES

Manuscript submitted: C. Gao and A. J. Sytkowski. Analysis of Constitutive Expression of the Erythropoietin Receptor Using a Tetracycline Inducible Expression Vector.

Manuscript In Review L. Feldman, Y. Wang, J.S. Rhim and A.J. Sytkowski. Human Prostate Cells Express Functional Erythropoietin Receptors.

Book Published: A. J. Sytkowski AJ. Erythropoietin: Blood Brain and Beyond. Wiley-VCH, Weinheim, 2004.

CONCLUSIONS

This second year of our work has seen excellent progress toward establishing our goals. We have prepared human prostate cancer cell lines with Epo receptor downregulated to be used in studies of growth and metastasis in SCID mice and have submitted a manuscript describing the "leaky" Tet-On vector. We completed 50 % of our immunohistochemistry study to identify prostate tumor cells expressing the human Epo receptor. and we completed baseline studies of human prostate cancer cell growth in vivo in SCID mice. Each of these initial accomplishments was necessary succeed in our Statement of Work tasks. The results of the second year imply success in achieving the goals proposed in this research.